Comparative Study of the Molecular Species of Chloropropanediol Diesters and Triacylglycerols in Milk Fat

5048

In an effort to establish the origin of the fatty acid esters of 3-chloropropanediol, which recently have been isolated in small amounts from goat milk, we compared the molecular species composition of the chlorohydrin diesters and of goat milk triacylglycerols. The chloropropanediol diesters were found to be composed of molecular species containing C_{10}–C_{18} fatty acids and corresponded closely in carbon number to those calculated for the long chain sn-1,2-diacylglycerol moieties of goat milk triacylglycerols. The molecular species of goat milk total triacylglycerols contained C_4–C_{18} fatty acids. It is suggested that triacylglycerols and chloropropanediol diesters are derived from the same pool of long chain fatty acids. A molecular distillate of bovine milk fat did not contain chloropropanediol diesters, while the available samples of human milk fat were shown to contain alkyldiacylglycerols as the major components of a neutral lipid fraction corresponding in polarity to the chloropropanediol diesters.
Lipids 21, 183–190 (1986).

During a detailed investigation of the lipids of goat milk, Cerbulis et al. (1,2) observed a neutral lipid fraction that migrated on thin layer chromatography (TLC) plates slightly ahead of the major triacylglycerols. Isolation and preliminary characterization of the fraction showed that it contained 3-chloro-1, 2-propanediol diacyl esters as major components (3). Similar compounds have been demonstrated independently by Gardner et al. (4) in adulterated Spanish cooking oils. In both instances the small amounts of the chloropropanediol diesters were isolated by preparative TLC from a large excess of triacylglycerols. The chemical nature of these substances was demonstrated by mass spectrometric identification of the chloropropanediol (3) or the [M-RCO_2] fragment ions of chloropropanediol diesters (4) by high resolution mass spectrometry.

Since Davidek et al. (5) previously had shown that monoesters and diesters of chloropropanediol may be produced by hydrolysis of triacylglycerols with HCl at 110 C, Gardner et al. (4) suggested that the chloropropanediol esters found in the adulterated Spanish cooking oil may have also been generated by exposure to HCl. These workers speculate that HCl may have been used to remove the aniline contained as a denaturant in the rapeseed oils used to adulterate the cooking oils. The goat milk, however, was fresh and had not been exposed to HCl at any time. The identification of the chloropropanediol diesters in fresh goat milk, therefore, excluded exposure to HCl as an explanation of their origin. The results of the present study are consistent with an esterification of either chloropropanediol or its monoester in the mamary gland.

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MATERIALS AND METHODS

The triacylglycerol and chloropropanediol diester fractions of goat milk were prepared as described by Cerbulis et al. (3). The analyzed sample represents material (Fig. 1) pooled from many TLC plates. Similar methods of isolation were used to obtain the corresponding fractions of triacylglycerols and chloropropanediol diesters from fresh samples of milk from six Philadelphia, Pennsylvania, mothers and from an Ontario cow. A large sample of the most volatile 2.5% of a molecular distillate of butterfat prepared in 1960 (6) was available in the laboratory and was also worked

FIG. 1. Thin layer chromatogram of the neutral lipid fractions of goat milk. The plate was silica gel G and the solvent was freshly prepared petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v). The arrow indicates the position of the fraction studied in this paper. Each track represents ca. 0.50 mg of lipid applied to the plate. The plates were charred with sulfuric acid/acetic acid/$FeCl_3$.

up as described by Cerbulis et al. (3). Rac-1-chloro-2, 3-dipalmitoyl-, rac-1-chloro-2,3-dioleoyl- and rac-1-chloro-2-palmitoyl-3-butyroylpropanediols were synthesized from rac-1-chloro-2,3-propanediol as described by Myher et al. (7). 1-Alkyl-2-palmitoyl 3-chloropropanediol was made from chimyl alcohol. 1-Alkyl-2,3-dipalmitoylglycerol was obtained by acylation of chimyl alcohol with palmitic anhydride in the presence of dimethylaminopyridine in benzene. The purified product then was digested with pancreatic lipase and the resulting 1-alkyl-2-palmitoyl glycerol was isolated by TLC. Conversion to the 3-chloro derivative was carried out by reacting this material with pyridine-phosphorus oxychloride (3:1) for one hr at 40 C.

Analysis of intact acylglycerols. Capillary gas liquid chromatography (GLC) of the acylglycerols was performed as previously described (8) on a Hewlett-Packard Model 5880A gas chromatograph (Hewlett-Packard Co., Palo Alto, California) equipped with an on-column injector and a fused silica capillary column (8 m × 0.32 mm I.D.) coated with SE-54 (Hewlett-Packard) using hydrogen as the carrier gas. The column temperature was programmed as indicated in the appropriate figures. Column bleed was minimal and was automatically subtracted via the single column compensation mode of the microprocessor terminal.

The high performance liquid chromatography (HPLC) analyses were performed with a Hewlett-Packard Model 1084B liquid chromatograph equipped with a Supelcosil LC-18 reversed phase column (Supelco, Bellefonte, Pennsylvania) using a gradient of 30-90% propionitrile in acetonitrile. The columns were operated at a flow rate of 1.5 ml/min and 30 C oven temperature. The mass spectrometry was done on a Hewlett-Packard Model 5985B quadrupole mass spectrometer equipped with a Hewlett-Packard direct liquid inlet interface and positive and negative chemical ionization detectors. The general layout of the liquid chromatography/mass spectroscopy (LC/MS) system and the methods of data analysis have been described previously (9,10). The mass spectrometer scans were taken every seven sec in the 200-900 mass range over the entire elution profile.

Analysis of alcohol moieties. Transmethylation of TLC purified natural lipids and of synthetic diesters of chloropropanediol was carried out as previously described (3); 50 μl of water was added and the mixture was extracted with petroleum ether. The remaining aqueous phase was neutralized with Dowex 1X8 in the hydroxyl form and decanted. The resin was washed with methanol, and the combined aqueous phase and methanol washings were evaporated. The resulting methanol/water mixture was analyzed by GLC; separation was by a modification of the method of Snyder and Franko-Filipasic (11). In this work a 1 m × 2 mm I.D. column of Tennax GC, previously conditioned under He at 300 C for 20 hr, was used on a Perkin Elmer Sigma 3B Chromatograph (Perkin Elmer Corp., Norwalk, Connecticut) with glass liner parts. The injector temperature was 240 C and the detector temperature was 300 C. Helium was the carrier gas at 45 ml/min, and the samples were eluted isothermally at 150 C. Detection was by flame ionization. Standard diols, triols and chloropropanediol were injected in a solvent made up of water/methanol (90:10, v/v). Under these conditions glycerol and chloropropanediol had retention times of 6.4 and 7.9 min, respectively.

The alkylglycerol moieties of alkyldiacylglycerols were determined by capillary GLC following acetylation of the transmethylation products, as previously described (12). The identities of the alkylglycerols were confirmed by chemical ionization MS using methane as reagent gas.

Analysis of fatty acid moieties. The fatty acid composition of the milk lipids was determined using either packed columns in combination with the n-butyl esters (6) or capillary columns in combination with the methyl esters (12).

RESULTS

Goat milk fat. Table 1 compares the fatty acid composition of the goat milk triacylglycerol and the chloropropanediol diesters. While the triacylglycerols contain significant amounts of both short and long chain acids (C_4–C_{18}), the chloropropanediol diesters possess largely long chain fatty acids (C_{10}–C_{18}). There is a great similarity in the relative content of the different unsaturated long chain fatty acids in the two acylglycerol fractions. In addition, both ester classes appear to possess comparable relative amounts of the odd carbon number fatty acids. The short chain fatty acids have either been excluded during the acylation of the chloropropanediol or the chloropropanediol esters of short and medium chain length fatty acids have not been resolved from the triacylglycerol bulk during the TLC separation. Interesting is the high content of stearic acid in the chloropropanediol diester fraction.

Capillary GLC on nonpolar columns was used to obtain detailed carbon number profiles for the triacylglycerol and the chloropropanediol diester fractions of goat milk. The carbon numbers of the

TABLE 1

Fatty Acid Composition of Goat Milk Triacylglycerols and Diacylchloropropanediols

Fatty acids	Triacylglycerols[a] (mol %)	Diacylchloropropanediols[b] (mol %)
4:0	3.5	
6:0	3.1	
8:0	3.2	
10:0	11.2	1.5
12:0	5.7	3.0
14:0	11.2	12.9
15:0	1.9	1.7
16:0	31.2	39.7
16:1 (n−7)	1.6	1.1
17:0	1.5	3.1
18:0	6.2	20.4
18:1 (n−9)	17.6	16.0
18:2 (n−6)	1.7	0.1
18:3 (n−3)	0.5	
20:0		0.5

[a]Goat milk fraction 5.

[b]Goat milk fraction 3.

triacylglycerols ranged from C_{28}–C_{54} and included small but significant amounts of species with odd carbon numbers. This pattern was similar to that reported by Cerbulis et al. (1) and Marai et al. (13), except that the odd carbon number species were not resolved by the columns used in the earlier studies. The carbon numbers of the chloropropanediols ranged from C_{26}–C_{38} and also included small amounts of species with odd carbon numbers. An accurate peak matching revealed that the latter esters were eluted together with the triacylglycerol of corresponding carbon numbers. For example, the synthetic dioleoyl chloropropanediol was eluted with an equivalent chain length of 37.9 relative to the set of triacylglycerols. Similarly, the butyroyl-palmitoylchloropropanediol was eluted slightly ahead of the retention time anticipated for a synthetic triacylglycerol with 24 acyl carbons. The goat milk fats examined in this study did not show significant peaks in the range of C_{16}–C_{28} triacylclycerols, which suggests that short chain fatty acid esters of chloropropanediol also were absent, although the very small peaks detected in this region were not examined by GC/MS. Table 2 gives the carbon number distributions for the two classes of esters of goat milk fat, along with that of another goat milk fat sample analyzed earlier (13). The earlier sample of goat milk triacylglycerols contains significantly more of the longer chain species (C_{50}–C_{54}) than the present sample. Table 3 compares the carbon number distribution of the chloropropanediol diesters with those of the sn-1,2- and the sn-2,3-diacylglycerol moieties as calculated from the known stereospecific fatty acid distribution of the earlier analyzed goat milk long chain triacylglycerol fraction. There is a close match between the carbon numbers of the chloropropanediol diesters and those of the sn-1,2- but not the sn-2,3-diacylglycerol moieties of the goat milk triacylglycerols.

Figure 2 compares the HPLC elution profiles of the goat milk triacylglycerols (lower panel) and of the

TABLE 2

Carbon Number Distribution of Goat Milk Triacylglycerols and Diacylchloropropanediols

Carbon numbers	Triacylglycerols (mol %)		Diacylchloro-propanediols[c] (mol %)
	Fraction 5[a]	Control[b]	
26			1.8
27			0.6
28			6.1
29			1.0
30	0.8	0.7	14.0
31			3.0
32			25.4
33	0.2	0.1	4.1
34	3.9	3.9	28.1
35	0.6	0.5	2.7
36	7.7	8.5	13.3
37	0.9	0.7	
38	10.8	12.4	
39	1.2	trace	
40	13.0	10.4	
41	0.4		
42	14.1	8.1	
43	1.6		
44	11.9	7.4	
45	1.5		
46	8.8	5.9	
47	0.9		
48	6.6	7.6	
49	0.9		
50	6.1	12.3	
51	0.5		
52	4.1	13.1	
53	0.3		
54	1.6	6.2	

[a]Goat milk fraction 5.

[b]Literature value (13).

[c]Goat milk fraction 3.

TABLE 3

Carbon Number Distribution of Diacylchloropropanediols and Diacylglycerol Moieties of Goat Milk Triacylglycerols

Carbon number	Diacylchloro-propanediols[a]	Diacylglycerol moieties (mol %)	
		sn-1,2-[b]	sn-2,3-[c]
26	1.8	3.3	6.8
27	0.6	0.2	0.3
28	6.1	5.6	7.7
29	1.0	0.7	0.7
30	14.0	14.1	6.3
31	3.0	2.4	1.8
32	25.4	28.2	16.1
33	4.0	2.4	3.5
34	28.1	27.8	27.9
35	2.7	0.6	1.4
36	13.3	11.2	19.0

[a]Goat milk fraction 3.

[b]1-random 2-random distribution calculated from literature data (13).

[c]2-random 3-random distribution calculated from literature data (13).

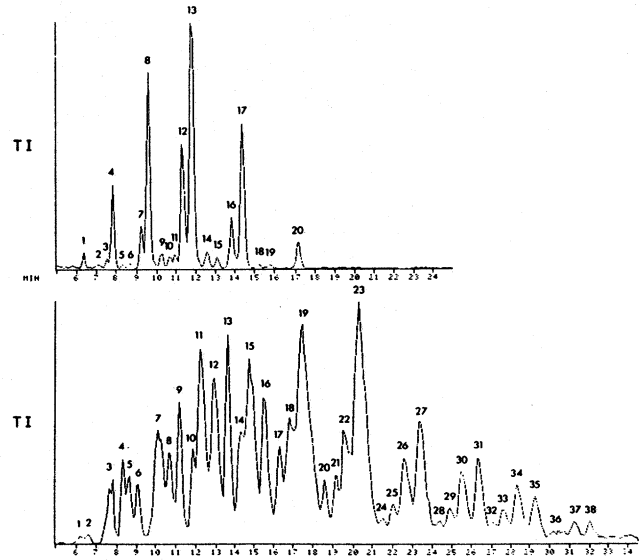

FIG. 2. HPLC elution profiles of the chloropropanediol diester (upper panel) and triacylglycerol (lower panel) fractions of goat milk fat. Chloropropanediol diester peaks are identified as in Table 4. LC/MS conditions as given in text.

chloropropanediol diesters (upper panel) as detected in the total positive ion current in the mass spectrometer. There is an extensive overlap between the two types of esters of corresponding partition number. Since the synthetic butyroylpalmitoylchloropropanediol was eluted well ahead of the short chain triacylglycerols, and in an area free of any other components in detectable amounts, it must be concluded that short chain esters of chloropropanediol were absent from the goat milk. This conclusion was confirmed by a search for characteristic positive and negative molecular and fragment ions over the anticipated range of elution times.

The chloropropanediol diester spectra obtained in the positive CI had $[MH\text{-}RCOOH]^+$ ions as the base peaks. The molecular weights of the esters were confirmed by the weak $[MH]^+$ ions. Thus, similar patterns were seen for the complete positive chemical ionization spectra of the synthetic dioleoylchloropropanediol and a major component (peak 12) from the HPLC elution profile (Fig. 2, upper panel) of the goat milk chlorohydrin esters. Because the synthetic compound had two identical fatty acids, only one mass (m/z 357) was seen for the $[MH\text{-}RCOOH]^+$ ion. The HPLC peak from the natural mixture apparently contained two different fatty acids in the same ester molecule, as indicated by the two different masses (m/z 357 and m/z 331) for the $[MH\text{-}RCOOH]^+$ ion. This was confirmed by the small pseudomolecular ion at m/z 613. These masses and fragment intensities were consistent with the presence of palmitoyloleoyl chloropropanediol as the sole component in this HPLC peak. The other chlorohydrin peaks showed the presence of two or more chloropropanediol diesters per peak. Table 4 lists the major ions detected in each of the chloropropanediol diester peaks. Table 5 gives the relative composition of the molecular species of the chloropropanediol diesters identified in the pooled goat milk sample. The major components are the dipalmitoyl, myristoylpalmitoyl, palmitoyloleoyl and palmitoylstearoyl species.

Figure 3 shows mass chromatograms corresponding to the m/z values of the MH^+ ions for the odd carbon-number diacylchloropropanediols. Because the major peaks elute with equivalent chain lengths (relative to the saturated diacylchloropropanediols) of 32.8, 34.8 and 36.8 instead of 29, 31 and 33, respectively, as expected for the odd carbon-numbered components, they must represent a new set of compounds. They were identified as 1-alkyl 2-acyl 3-chloropropanediols. Figure 4 shows the spectrum corresponding to one of the molecular species found in the natural sample along with the spectrum of a synthetic standard. Both the mass spectra and the chromatographic retention times are consistent with the proposed identity of these compounds.

The positive chemical ionization spectra of the goat milk fat triacylglycerols were similar to those recorded for other natural mixed-acid triacylglycerols (14). Prominent intensities were recorded for the protonated molecular ions $[MH]^+$, along with low abundance ions corresponding to adducts of acetonitrile and propionitrile. The $[MH\text{-}RCOOH]^+$ ions resulting from a random loss of an acid moiety from the protonated molecular ion were responsible for the base peaks in most of

TABLE 4

Major Ions[a] in the Chemical Ionization Spectra of Diacylchloropropanediols as obtained by LC/MS with a Gradient of Propionitrile in Acetonitrile

HPLC Peak	$(MH\text{-}RCOOH)^+$ (m/z)	$(MH)^+$ (m/z)	Major species[b] of diacylchloropropanediols
1	247, 275, 303, 331	503	10:0 16:0, 12:0 14:0
2	247, 331	503	10:0 16:0
3	275, 357	557	12:0 18:1, 14:0 16:1
4	275, 303, 331, 359	531	12:0 16:0, 14:0 14:0
			10:0 18:0
5	275, 289, 303, 317	545	14:0 15:0, 12:0 17:0
6	275, 289, 303, 317	545	14:0 15:0, 12:0 17:0
7	303, 357	585	14:0 18:1
8	275, 303, 331, 359	559	14:0 16:0, 12:0 18:0
9	303, 317, 331, 345	573	14:0 17:0, 15:0 16:0
10	303, 317, 331, 345	573	14:0 17:0, 15:0 16:0
11	357	639	18:1 18:1
12	331, 357	613	16:0 18:1
13	303, 331, 359	587	16:0 16:0, 14:0 18:0
13a	345, 357	627	17:0 18:1
14	317, 331, 345, 359	601	16:0 17:0, 15:0 18:0
	345, 357	627	17:0 18:1
15	317. 331, 345, 359	601	16:0 17:0, 15:0 18:0
16	357, 359	641	18:0 18:1
18	331, 359	615	16:0 18:0
17	331, 345, 359, 373	629	17:0 18:0, 19:0 16:0
19	345, 359	629	17:0 18:0
20	359	643	18:0 18:0
21	331, 387, 415	671	18:0 20:0, 16:0 22:0

[a]The identity of the ions is described in text.

[b]No distinction is being made among the sn-2 and sn-3 positions of the diacylchloropropanediol molecule.

TABLE 5

Relative Proportions of Molecular Species of Diacylchloropropanediols in a Pooled Sample of Goat Milk Fat[a]

HPLC peak	Molecular species	Area %	HPLC peak	Molecular species	Area %
1	10:0 16:0	0.8	10	14:0 17:0	0.7
	12:0 14:0	0.2		15:0 16:0	0.6
2	10:0 16:0	0.6	11	18:1 18:1	1.4
3	12:0 18:1	0.8	12	16:0 18:1	12.6
4	12:0 16:0	3.3	13	16:0 16:0	23.5
	14:0 14:0	1.7		14:0 18:0	3.2
	10:0 18:0	1.6	14	16:0 17:0	2.0
5	14:0 15:0	0.2	15	16:0 17:0	0.6
	12:0 17:0	0.2		15:0 18:0	0.5
6	14:0 15:0	0.2	16	18:0 18:1	5.1
	12:0 17:0	0.2	17	16:0 18:0	14.4
7	14:0 18:1	3.7	18	17:0 18:0	0.4
8	14:0 16:0	14.6		19:0 16:0	0.1
	12:0 18:0	1.6	19	17:0 18:0	0.5
9	14:0 17:0	0.8	20	18:0 18:0	2.8
	15:0 16:0	0.8	21	18:0 20:0	0.1

[a]Molecular species making up less than 10% of the HPLC peak have been ignored, with the peak area being divided among the identified species.

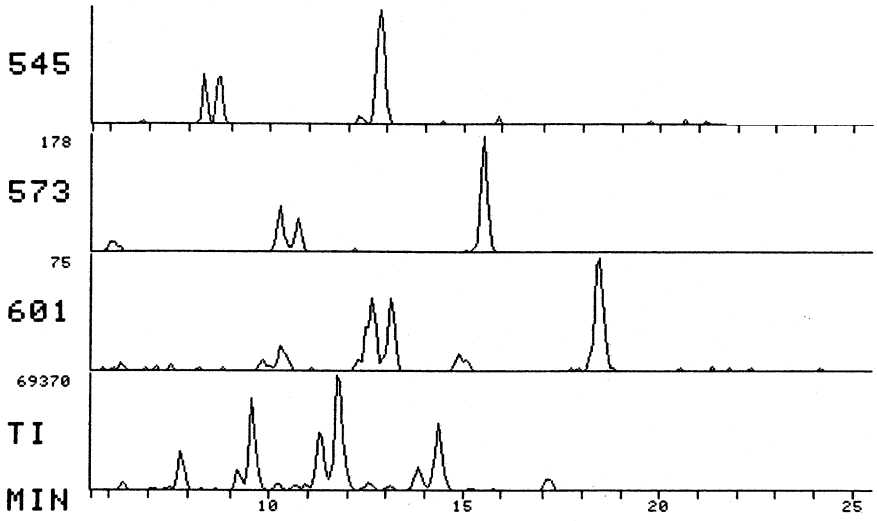

FIG. 3. Mass chromatograms of diacylchloropropanediols showing m/z values corresponding to MH^+ ions for odd carbon number species. The major peaks at m/z 545, 573 and 601 constitute a separate series of homologous species identified as alkylacylchloropropanediols.

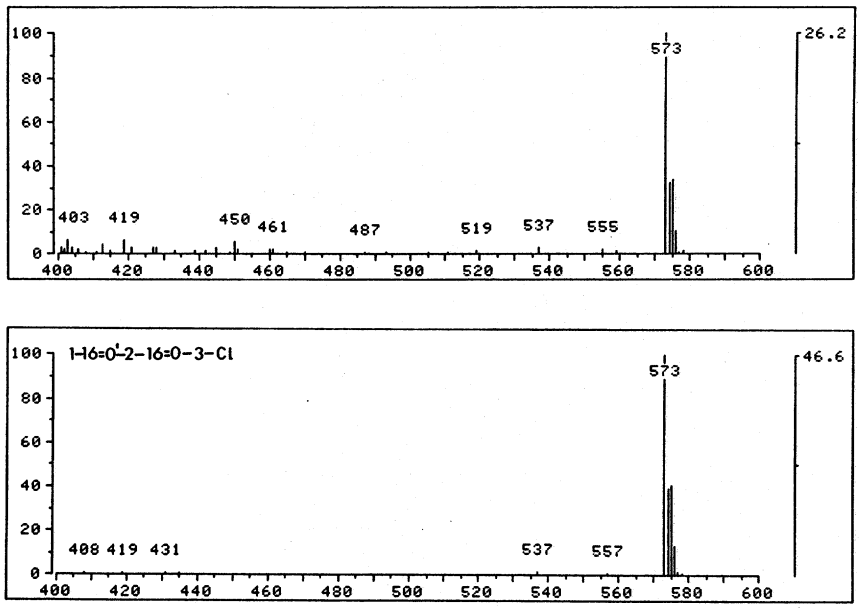

FIG. 4. Chemical ionization spectra of synthetic 1-palmityl 2-palmitoyl 3-chloropropanediol (lower panel) and one of the unknown series of species found in the natural sample of goat milk fat (upper panel).

these spectra. Using the LC/MS positive chemical ionization approach, it was possible to estimate that the short chain esters of chloropropanediol, if present, could not have exceeded 10% of the long chain chloropropanediol diesters in the goat milk.

Bovine milk fat. Figure 5 shows the total ion current profile of an LC/MS separation of a molecular distillate of bovine milk fat along with the pattern of the chloropropanediol diesters isolated from goat milk. Although there is an extensive overlap among the various components, a positive identification of any chloropropanediol diesters in the milk fat distillate cannot be made on the basis of the positive chemical ionization spectra of the components. A positive identification of these esters also could not be obtained by capillary GLC of the distillate or of the TLC fractions of the molecular distillate corresponding in migration rate to synthetic butyroylpalmitoyl and butyroyloleoylchloropropanediols.

To increase the specificity of detection of the chloropropanediol diesters, we attempted LC/MS with negative ion detection. Figure 6 shows the negative ion

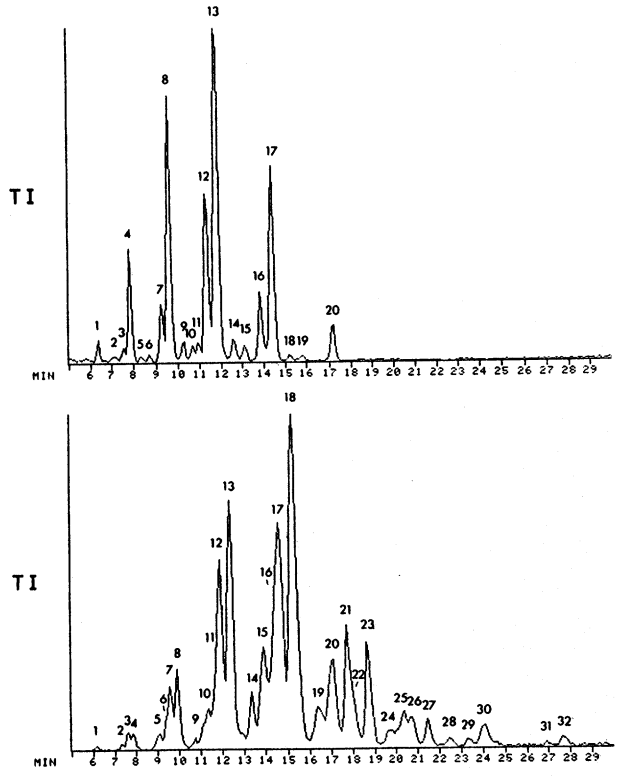

FIG. 5. HPLC elution profiles of the chloropropanediol diesters of goat milk (upper panel) and the 2.5% most volatile molecular distillate of bovine milk fat (lower panel). The chloropropanediol diester peaks are identified as in Table 4. The identity of the major peaks in the molecular distillate is as follows: 7—12:0, 18:1, 4:0; 8—14:0,14:0,4:0; 12—14:0, 18:1, 4:0 + 14:0, 14:0, 6:0; 13—14:0, 16:0, 4:0; 15—14:0, 18:1, 6:0 + 10:0, 12:0, 14:0; 17—16:0,18:1, 4:0 + 16:0, 14:0, 6:0; 18—16:0, 16:0, 4:0; 19—16:0, 17:0, 4:0; 20—16:0, 18:1, 12:0, 8:0; 21—16:0, 16:0, 6:0; 23—18:0, 16:0, 4:0; 25—16:0, 14:0, 10:0,; 27—18:0, 16:0, 6:0.

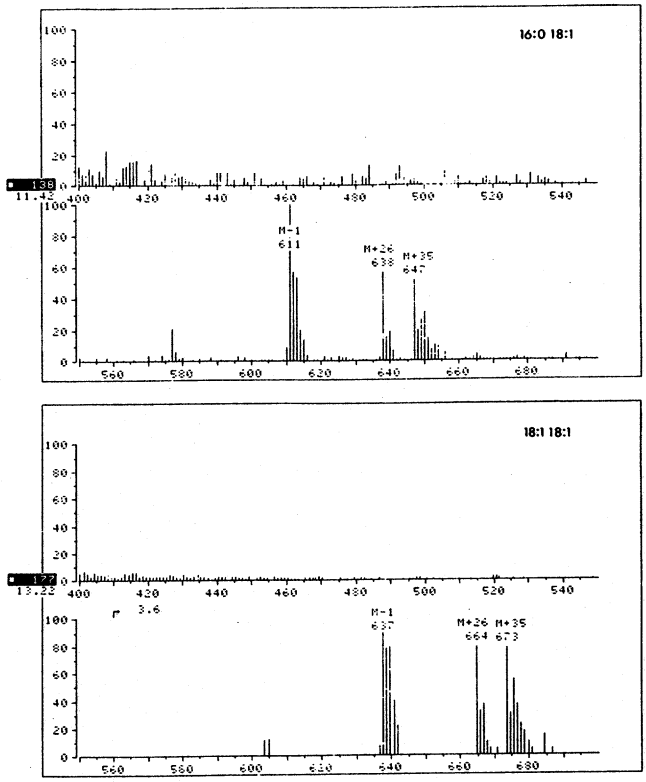

FIG. 6. Negative chemical ionization spectra of the natural palmitoyloleoylchloropropanediol (upper panel) and synthetic dioleoylchloropropanediol (lower panel) as obtained under LC/MS conditions using a gradient of propionitrile in acetonitrile. LC/MS conditions as given in text.

chemical ionization spectra for the dipalmitoylchloropropanediol (upper panel) recovered from the goat milk sample (peak 12, Fig. 2, upper panel) and the synthetic dioleoylchloropropanediol (lower panel). The spectra obtained in the negative CI mode exhibited ions at m/z $[M-1]^+$, $[M+26]^+$ and $[M+35]^+$. The latter two ions represent the addition of CN^- and Cl^- ions, respectively. The isotope clusters of the ions at m/z $[M+26]^+$ and $[M+35]^+$ are consistent with the presence of one and two chlorine atoms, respectively. The negative chemical ionization detection provided characteristic fragment ions not found in the ordinary triacylglycerol spectra. An examination of the distillate fraction by this method, however, gave negative results for chloropropanediol diesters in the 2.5% distillate of the butterfat. Likewise, neither the original triacylglycerol mixture nor the unknown fatty esters recovered from the front of the triacylglycerol spot on the TLC plate gave ions characteristic of the chloropropanediol diesters in either positive or negative chemical ionization mass spectra.

Human milk fat. Figure 7 compares the capillary GLC elution patterns of human milk triacylglycerols (lower panel) and of the fatty acid esters recovered from a TLC spot corresponding to the goat milk chloropropanediol diesters (upper panel). The unknown esters of the human milk are of higher molecular weight than the chloropropanediol diesters. These esters give an elution pattern similar to that obtained for human milk triacylglycerols under the same GLC conditions, but the major peaks are eluted somewhat earlier than the anticipated major triacylglycerol peaks. Such a behavior is characteristic of the alkyldiacylglycerols compared to triacylglycerols of corresponding carbon number. The presence of the alkyldiacylglycerols in the minor neutral lipid fraction of human milk was confirmed by capillary GLC of the acetylated transmethylation products, which showed the presence of both fatty acid methyl esters and of the diacetates of palmityl, stearyl and oleylglycerols as major components. The composition is shown in Table 6. Furthermore, the unknown fatty ester fraction of human milk failed to produce any of the positive or negative chemical ionization products seen in the LC/MS spectra of the goat milk chloropropanediol diesters.

Ten other human milk fat samples were examined by TLC. Each milk contained a TLC spot corresponding to the chloropropanediol diesters. Enough material was isolated from five sources to attempt to determine if the chloropropanediol diesters were present. The TLC spots were transmethylated as previously described (3), and the aqueous phase was recovered and analyzed

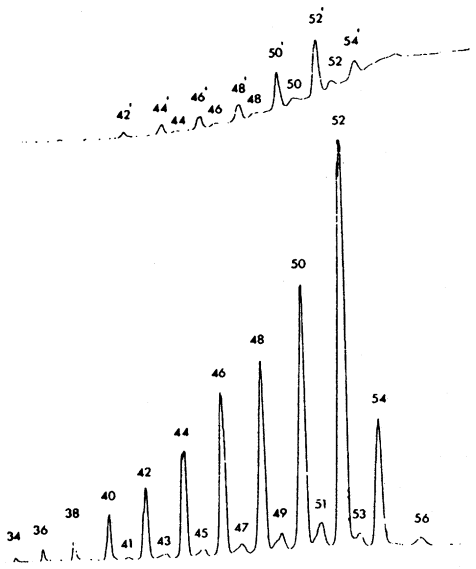

FIG. 7. Capillary GLC elution patterns of the alkyldiacylglycerol (upper panel) and the triacylglycerol (lower panel) of human milk fat. The triacyglycerol peaks are identified by the number of total acyl carbons and the alkyldiacylglycerols by the number of total fatty chain carbons per acylglycerol molecule. GLC conditions as given in text.

TABLE 6

Composition of Alkyldiacylglycerols, Triacylglycerols and Monoalkylglycerols from Human Milk

	Fatty acid methyl esters (mol %)		
Chain length	Alkyldiacyl-glycerols	Triacyl-glycerols	Monoalkylgly-cerols (mol %)
10:0 & shorter		3.9	
12:0	3.3	8.2	
14:0	6.6	8.3	
15:0	0.4	0.5	
16:0	34.0	24.2	23.9
16:1	3.2	3.1	
17:0	0.5	0.4	1.7
18:0	8.8	10.3	43.3
18:1	35.6	34.7	31.1
18:2	5.0	5.6	
20:0	1.2	0.6	
20:1	1.4	0.4	

Ca. 7% of the ether fraction contained alkenyl groups having either 16 or 18 carbon atoms.

by GLC for the presence of 3-chloropropanediol. Only three samples showed the presence of 3-chloropropanediol as judged by retention times, and in these cases it accounted for less than 5% of the total recovered alcohols. The major component in each case was glycerol, and a number of other minor components with unidentified retention times were observed. Thus, chloropropanediol diesters are not a major component of the corresponding TLC spot in human milk.

DISCUSSION

Our results confirm the presence of fatty acid esters of chloropropanediol in goat milk fat, from which they have been isolated as a minor TLC fraction migrating just ahead of the triacylglycerols in a neutral lipid solvent system. Although similar neutral lipid spots have been noted for milk fat samples from other herds of goats, it is not possible to conclude that the chloropropanediol diesters are characteristic components of goat milk fat. Detailed analyses of the corresponding fractions from human and bovine milk fat revealed the alkyldiacylglycerols as the major or sole components. It is therefore essential to confirm in each instance the presence of the chloropropanediol diesters by chromatographic and mass spectrometric means before the origin of these compounds is considered.

LC/MS of milk fats. The present study constitutes the first practical application of LC/MS to the study of the acylglycerol composition of the highly complex ruminant milk fats, although the suitability of the method has been demonstrated previously (14,15). Of special interest is the resolution of the short and medium chain triacylglycerols within a carbon number. Both specific molecular association and positional distribution of the fatty acids apparently contribute to their partition on the reversed-phase HPLC column as well as to the relative retention time on a polar GLC column (16). Carbon or partition numbers cannot be used to characterize and identify these molecules by HPLC. Accurate peak collection and analysis of fatty acid composition might help, but the necessary confidence in the correctness of the results can be obtained only by MS.

The LC/MS also allows the detection and identification of nonglyceride components that may contaminate the triacylglycerol fraction. We have previously described the overlaps between triacylglycerols and the fatty acid esters of cholesterol and plant sterols (14,15), which can be effectively demonstrated by mass chromatography. The present study shows that mass chromatography can also be used to demonstrate the presence of other minor components in the milk fat, provided the masses of the potential contaminants are accurately known and do not coincide with the masses of the major triacylglycerol ions or their P+1 and P+2 companions. In view of the extreme complexity of the milk fat triacylglycerols, however, the levels of contaminants that can be detected without prior enrichment by TLC or molecular distillation are rather high, except in those instances where the contaminants are clearly resolved from the bulk of the triacylglycerols by the reversed-phase HPLC column. The long chain diesters of the chloropropanediol and the long chain alkyldiacylglycerols require a preliminary isolation. The long chain alkyldiacylglycerols have been previously detected in both human and bovine milk fats (17). The present study shows that on TLC these migrate to the same spot as the long chain fatty acid esters of chloropropanediol.

Identification of chloropropanediol diesters. Electron impact spectra of chloropropanediol diesters have been obtained previously by Velisek et al. (18), Davidek et al. (5) and Gardner et al. (4), who identified fragments

characteristic of the fatty acid components, $[RCO]^+$, and of the loss of fatty acids from the parent molecule, $[M\text{-}RCOO]^+$. The presence of chlorine in the $[M\text{-}RCOO]^+$ fragment ions was confirmed by high resolution EIMS (4).

The present study confirms the presence of the molecular species of carbon numbers C_{26}–C_{38} identified in the chloropropanediol diesters of goat milk by Cerbulis et al. (3) on the basis of the fatty acid composition and the molecular weight estimates derived by ammonia/direct chemical ionization analyses. The isolated chloropropanediol diester fraction indicated the presence of nine major components with molecular weights of 502, 528, 530, 556, 558, 584, 586, 612 and 614. Compounds with molecular weights of 638, 640 and 642 also were observed in the ammonia/direct chemical ionization analyses, although intensities were low. The quasi-molecular ions of these compunds showed characteristic isotopic patterns of one chlorine atom. The LC/MS analysis allowed a complete identification of the fatty acid pairing within each carbon number, as well as a matching of the obtained pattern to that derived for the long chain sn-1,2-diacylglycerol moieties of the goat milk triacylglycerols. No evidence was obtained for the presence of short chain diesters of chloropropanediol in the goat milk fat.

Origin of fatty esters of chloropropanediol. In view of the close similarity in composition of the molecular species of the chloropropanediol diesters and the sn-1,2-diacylglycerol moieties of goat milk triacylglycerols, it would appear that a chlorination of the hydroxyl group had taken place after the formation of the sn-1,2-diacylglycerol intermediates of triacylglycerol biosynthesis. This also is consistent with the finding of the 3-chloro-3-deoxyderivative of 1-alkyl 2-acylglycerol known to be synthesized by a stereochemically specific mechanism involving dihydroxyacetone phosphate. Furthermore, this possibility is likely in view of the absence of the short chain esters of chloropropanediol. The short chain fatty acids are believed to be introduced into the 3-position of the sn-glycerol molecule as the final acylation step in the biosynthesis of milk fat triacylglycerols (19). Although a coincidence cannot be ruled out, the present results would appear to justify a further comparative stereospecific study of the chloropropanediol diesters and of the triacylglycerols collected from the same goat milk sample (7).

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